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54 The class II protein of the outer membrane of *neisseria meningitidis*.

57 The Class II major immuno-enhancing protein (MIEP) of *Neisseria meningitidis*, purified directly from the outer membrane of *Neisseria meningitidis*, or obtained through recombinant cloning and expression of DNA encoding the MIEP of *Neisseria meningitidis*, has immunologic carrier as well as immunologic enhancement and mitogenic properties.

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BACKGROUND OF THE INVENTION

The outer membrane protein complex (OMPC) of Neisseria meningitidis is used as an immunologic carrier in vaccines for human use. OMPC consists of vesicles containing a variety of proteins as well as membranous lipids, including lipopolysaccharide (LPS or endotoxin).

OMPC has the property of immune enhancement, and when an antigen is chemically coupled to it, an increased antibody response to the antigen results. OMPC is currently used in vaccines for human infants against infectious agents such as Haemophilus influenzae, and renders the infants capable of mounting an IgG and memory immune response to polyribosyl ribitol phosphate (PRP) of H. influenzae, when PRP is chemically coupled to OMPC.

OMPC is a mixture of a variety of proteins and lipids, and it was not known which component or components of OMPC bestows the beneficial immune enhancing effect to the coupled antigens. However, some potentially negative aspects of using OMPC in human vaccines include LPS related reactions such as fever, endotoxic shock, hypotension, neutropenia, activation of the alternative complement pathway, intravascular coagulation, and possibly death.

Furthermore, OMPC-antigen conjugates are quite heterogeneous in that the antigen may become conjugated to any of the protein moieties which make up OMPC.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide substantially pure Class II, major immuno enhancing protein (MIEP) derived directly from the outer membrane of Neisseria meningitidis, free from other Neisseria meningitidis outer membrane components. It is another object of the present invention to provide substantially pure recombinant MIEP of the outer membrane of Neisseria meningitidis, produced in a recombinant host cell, completely free of all other Neisseria meningitidis proteins. A further object of the present invention is to provide an efficient immunocarrier protein for the enhancement of an immune response to antigens, comprising either MIEP purified directly from the outer membrane of Neisseria meningitidis, or recombinant MIEP of Neisseria meningitidis produced in a recombinant host cell. Another object of the present invention is to provide a protein which possesses immune mitogenic activity, comprising either MIEP purified directly from the outer membrane of Neisseria meningitidis, or recombinant MIEP of Neisseria meningitidis produced in a recombinant host cell. Another object of the present invention is to provide a protein which possesses the ability to induce the production of cytokines such as I1-2, comprising either MIEP purified directly from the outer membrane of Neisseria meningitidis, or recombinant MIEP of Neisseria meningitidis produced in a recombinant host cell.

An additional object of the present invention is to provide vaccine compositions containing either the recombinant MIEP, or MIEP purified directly from the outer membrane of Neisseria meningitidis. These and other objects will be apparent from the following description.

SUMMARY OF THE INVENTION

The present invention relates to the Class II major immuno enhancing protein (MIEP) of the outer membrane of Neisseria meningitidis, in substantially pure form, free from other contaminating N. meningitidis outer membrane proteins and LPS. The MIEP of the present invention, whether purified directly from the outer membrane of Neisseria meningitidis cells, or derived from a recombinant host cell producing recombinant MIEP of Neisseria meningitidis, possesses immunologic carrier cytokine (I1-2) inducing activity and mitogenic activity. The MIEP of the present invention, when coupled to an antigen, is capable of immune enhancement in that the antibody response to the coupled antigen is augmented or the antigen is transformed to a T-dependent antigen which ensures that immunoglobulins of the IgG class are produced. The antigens which may be coupled to the MIEP of the present invention include viral proteins, bacterial proteins and polysaccharides, synthetic peptides, other immunogenic antigens, and weak or non-immunogenic antigens.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 - Antibody responses of adoptive transfer recipients receiving spleen cells primed separately with PRP-DT and MIEP, or OMPC, or IAA-OMPC, were measured by ELISA and RIA in blood samples taken on the indicated days post-immunization with PRP-OMPC.

Figure 2 - Lymphocyte proliferation assay for mitogenic activity of MIEP, in vitro. The increase in ³H-thymidine incorporation into cellular DNA was measured following exposure of the cells to bovine serum albumin (BSA), PRP-OMPC, OMPC, MIEP, or CNBr.

meningitidis is a porin protein [Murakami, K., et al., (1989), *Infection And Immunity*, 57, pp.2318-23]. Porins are found in the outer membrane of all Gram negative bacteria.

While the present invention is exemplified by MIEP of N. meningitidis, it is readily apparent to those skilled in the art that any outer membrane protein from any Gram negative bacterium, which has immunologic carrier and immune enhancement activity, is encompassed by the present invention. Examples of Gram negative bacteria include but are not limited to species of the genera Neisseria, Escherichia, Pseudomonas, Hemophilus, Salmonella, Shigella, Bordetella, Klebsiella, Serratia, Yersinia, Vibrio, and Enterobacter.

MIEP may be employed to potentiate the antibody response to highly antigenic, weakly antigenic, and non-antigenic materials. The term "antigen" and "antigenic material" which are used interchangeably herein include one or more non-viable, immunogenic, weakly immunogenic, non-immunogenic, or desensitizing (antiallergic) agents of bacterial, viral, or other origin. The antigen component may consist of a dried powder, an aqueous phase such as an aqueous solution, or an aqueous suspension and the like, including mixtures of the same, containing a non-viable, immunogenic, weakly immunogenic, non-immunogenic, or desensitizing agent or agents.

The aqueous phase may conveniently be comprised of the antigenic material in a parenterally acceptable liquid. For example, the aqueous phase may be in the form of a vaccine in which the antigen is dissolved in a balanced salt solution, physiological saline solution, phosphate buffered saline solution, tissue culture fluids, or other media in which an organism may have been grown. The aqueous phase also may contain preservatives and/or substances conventionally incorporated in vaccine preparations. Adjuvant emulsions containing MIEP conjugated antigen may be prepared employing techniques well known to the art.

The antigen may be in the form of purified or partially purified antigen including but not limited to antigens derived from bacteria, viruses, mammalian cells, fungi, rickettsia; or the antigen may be an allergen including but not limited to pollens, dusts, danders, or extracts of the same; or the antigen may be in the form of a poison or a venom including but not limited to poisons or venoms derived from poisonous insects or reptiles. The antigen may also be in the form of a synthetic peptide, or a fragment of a larger polypeptide, or any subportion of a molecule or component derived from bacteria, mammalian cell, fungi, viruses, rickettsia, allergen, poison or venom. In all cases, the antigens will be in the form in which their toxic or virulent properties have been reduced or destroyed and which when introduced into a suitable host will either induce active immunity by the production therein of antibodies against the specific proteins, peptides, microorganisms, extract, or products of microorganisms used in the preparation of the antigen, poisons, venoms, or, in the case of allergens, they will aid in alleviating the symptoms of the allergy due to the specific allergen.

The antigens can be used either singly or in combination, for example, multiple bacterial antigens, multiple viral antigens, multiple mycoplasmal antigens, multiple rickettsial antigens, multiple bacterial or viral toxoids, multiple allergens, multiple proteins, multiple peptides or combinations of any of the foregoing products can be conjugated to MIEP.

Antigens of particular importance are derived from bacteria including but not limited to B. pertussis, Lep-tospira pomona, and icterohaemorrhagiae, S. paratyphi A and B, C. diphtheriae, C. tetani, C. botulinum, C. per-fringens, C. fesi, and other gas gangrene bacteria, B. anthracis, P. pestis, P. multocida, V. cholerae, Neisseria meningitidis, N. gonorrhoeae, Hemophilus, Influenzae, Treponema pallidum, and the like; from mammalian cells including but not limited to tumor cells, virus infected cells, genetically engineered cells, cells grown in culture, cell or tissue extracts, and the like; from viruses including but not limited to human T lymphotropic virus (multiple types), human immunodeficiency virus (multiple variants and types), polio virus (multiple types), adeno virus (multiple types), parainfluenza virus (multiple types), measles, mumps, respiratory syncytial virus, influenza virus (various types), shipping fever virus (SF₄), Western and Eastern equine encephalomyelitis virus, Japanese B. encephalomyelitis, Russian Spring-Summer encephalomyelitis, hog cholera virus, Newcastle disease virus, fowl pox, rabies, feline and canine distemper and the like viruses, from rickettsiae including but not limited to epidemic and endemic typhus or other members of the spotted fever group, from various spider and snake venoms or any of the known allergens, including but not limited to those from ragweed, house dust, pollen extracts, grass pollens, and the like.

The polysaccharides of this invention may be any bacterial polysaccharides with acid groups, but are not intended to be limited to any particular types. Examples of such bacterial polysaccharides include Streptococcus pneumoniae (pneumococcal) types 6A, 6B, 10A, 11A, 18C, 19A, 19f, 20, 22F, and 23F, polysaccharides; Group B Streptococcus types Ia, Ib, II and III; Haemophilus influenzae serotype b polysaccharide; Neisseria meningitidis serogroups A, B, C, X, Y, W135 and 29E polysaccharides; and Escherichia coli K1, K12, K13, K92 and K100 polysaccharides. Particularly preferred polysaccharides, however, are those capsular polysaccharides selected from the group consisting of H. influenzae serotype b polysaccharides, such as described in Rosenberg et al., *J. Biol. Chem.*, 238, 2845-2849 (1961) and Zamenhof et al., *J. Biol. Chem.*, 203, 695-704 (1953). Streptococcus pneumoniae (pneumococcal) type 6B or type 6A polysaccharide, such as described in

Robbins et al., *Infection and Immunity*, 26, No. 3 1116-1122 (Dec., 1979); pneumococcal type 19F polysaccharide, such as described C. J. Lee et al., *Reviews of Infectious Diseases*, 3, No. 2, 323-331 (1981); and pneumococcal type 23F polysaccharide, such as described in O. Larm et al., *Adv. Carbohydr Chem and Biochem.*, 33, 295-321, R. S. Tipson et al., ed., Academic Press 1976.

MIEP can be purified from OMPC derived from cultures of *N. meningitidis* grown in the usual manner as described in U.S. Patent number 4,459,286 and U.S. Patent number 4,830,852. OMPC purification can be done according to the methods described in U.S. Patent number 4,271,147, 4,459,286, and 4,830,852.

MIEP can also be obtained from recombinant DNA engineered host cells by expression of recombinant DNA encoding MIEP. The DNA encoding MIEP can be obtained from *N. meningitidis* cells [Murakami, K. et al., (1989), *Infection And Immunity*, 57, pp. 2318], or the DNA can be produced synthetically using standard DNA synthesis techniques. DNA encoding MIEP can be expressed in recombinant host cells including but not limited to bacteria, yeast, insect, mammalian or other animal cells, yielding recombinant MIEP. The preferred methods of the present invention for obtaining MIEP are purification of MIEP from OMPC and recombinant DNA expression of DNA encoding MIEP derived from *N. meningitidis*, with purification from OMPC most preferred.

Purified MIEP was prepared from OMPC vesicles by sodium dodecylsulfate (SDS) lysis of the vesicles followed by SDS polyacrylamide gel electrophoresis (PAGE). The MIEP was eluted from the gel, dialysed against a high pH buffer and concentrated. Standard methods of polyacrylamide gel electrophoresis can be utilized to purify MIEP from OMPC vesicles. Such methods are described in *Molecular Cloning: A Laboratory Manual*, Sambrook, J. et al., (1989), Cold Spring Harbor Laboratory Press, New York, and *Current Protocols In Molecular Biology*, (1987) Ausubel F.M. et al., editors, Wiley and Sons, New York.

Standard methods of eluting proteins from SDS-polyacrylamide gels are described in Hunkapiller, M.W., and Lujan, E., (1986), *Purification Of Microgram Quantities Of Proteins By Polyacrylamide Gel Electrophoresis*, in *Methods of Protein Microcharacterization* (J. Shively editor) Humanna Press, Clifton N.J., and *Current Protocols In Molecular Biology* (1987), Ausubel, F.M., et al., editors, Wiley and Sons, New York.

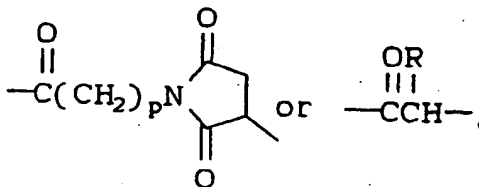
MIEP prepared in this manner is readily suitable for conjugation to antigens derived from bacteria, viruses, mammalian cells, rickettsia, allergens, poisons or venoms, fungi, peptides, proteins, polysaccharides, or any other antigen.

Recombinant MIEP can be prepared by expression of genomic *N. meningitidis* DNA encoding MIEP in bacteria, for example *E. coli* or in yeast, for example *S. cerevisiae*. To obtain genomic DNA encoding MIEP, genomic DNA is extracted from *N. meningitidis* and prepared for cloning by either random fragmentation of high molecular weight DNA following the technique of Maniatis, T. et al., (1978), *Cell*, 15, pp. 687, or by cleavage with a restriction endonuclease by the method of Smithies, et al., (1978), *Science*, 202, pp. 1248. The genomic DNA is then incorporated into an appropriate cloning vector, for example lambda phage [see Sambrook, J. et al., (1989), *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Press, New York]. Alternatively, the polymerase chain reaction (PCR) technique (Perkin Elmer) can be used to amplify specific DNA sequences in the genomic DNA [Roux, et al., (1989), *Biotechniques*, 8, pp. 48]. PCR treatment requires a DNA oligonucleotide which can hybridize with specific DNA sequences in the genomic DNA. The DNA sequence of the DNA oligonucleotides which can hybridize to MIEP DNA in the *N. meningitidis* genomic DNA can be determined from the amino acid sequence of MIEP or by reference to the determined DNA sequence for the Class II major membrane protein of *N. meningitidis* [Musakami, k. et al., (1989), *Infection and Immunity*, 57, pp. 2318].

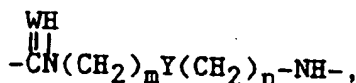
Recombinant MIEP can be separated from other cellular proteins by use of an affinity column made with monoclonal or polyclonal antibodies specific for MIEP. These affinity columns are made by adding the antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing MIEP are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The protein is then dialyzed against phosphate buffered saline.

The conjugates of the present invention may be any stable polysaccharide-MIEP conjugates, or any other antigen-MIEP conjugates, including synthetic peptide antigens. The synthetic peptides may possess one or more antigenic determinants of any antigen including antigenic determinants from bacteria, rickettsia, viruses (including human immunodeficiency viruses), mammalian cells or other eukaryotic cells including parasites, toxins or poisons, or allergens. The antigen-MIEP conjugates are coupled through bigeneric spacers containing a thioether group and primary amine, which form hydrolytically-labile covalent bonds with the polysaccharide and the MIEP. Preferred conjugates according to this invention, however, are those which may be represented

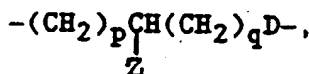
by the formulae, Ps-A-E-S-B-Pro or Ps-A'-S-E'-B'-Pro, wherein Ps represents a poly-saccharide or any other antigen; Pro represents the bacterial protein MIEP; and A-E-S-B and A'-S-E'-B' constitute bigeneric spacers which contain hydrolytically-stable covalent thioether bonds, and which form covalent bonds (such as hydrolytically-labile ester or amide bonds) with the macromolecules, Pro and Ps. In the spacer, A-E-S-B, S is sulfur; E is the transformation product of a thiophilic group which has been reacted with a thiol group, and is represented by



wherein R is H or CH₃, and p is 1 to 3; A is



wherein W is O or NH, m is 0 to 4, n is 0 to 3, and Y is CH₂, O, S, NR', or CHCO₂H, where R' is H or C₁- or C₂-alkyl, such that if Y is CH₂, then both m and n cannot equal zero, and if Y is O or S, then m is greater than 1 and n is greater than 1; and B is



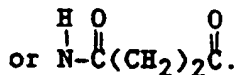
wherein q is 0 to 2, Z is NH₂,



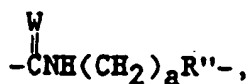
COOH, or H, where R' and p are as defined above, and D is



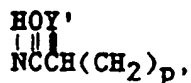
NR',



Then in the spacer, A'-S-E'-B', S is sulfur; A' is



wherein a is 1 to 4, and R'' is CH₂, or



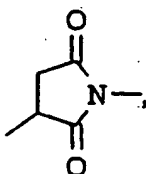
5 where Y' is NH₂ or NHCOR', and W, p and R' are as defined above, and E' is the transformation product of a thiophilic group which has been reacted with a thiol group, and is represented by



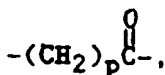
10 wherein R is as defined above, and B' is



15 or E' is



20 B' is



25 wherein p is 1 to 3. Further, of the bigeneric spacers, A-E-S-B and A'-S-E'-B', the E-S-B and A'-S-E' components are determinable and quantifiable, with this identification reflecting the covalency of the conjugate bond linking the side of the thioethersulfur which originates from the covalently-modified polysaccharide with the side of the spacer which originates from the functionalized protein.

30 Then the conjugates, Ps-A-E-S-B-Pro, according to this invention may contain spacers whose components include derivatives of, *inter alia*: carbon dioxide, 1,4-butanediamine, and S-carboxymethyl-N-acetylhomocysteine; carbon dioxide, 1,5-pentanediamine, and S-carboxymethyl-N-acetylhomocysteine; carbon dioxide, 3-oxa-1,5-pentanediamine, and S-carboxymethyl-N-acetylhomocysteine; carbon dioxide, 1,4-butane-diamine, and S-carboxymethyl-N-acetylcysteine; carbon dioxide, 1,3-propanediamine, and S-carboxymethyl-N-benzoylhomocysteine; carbon dioxide, 3-aza-1,5-pentanediamine, and S-carboxymethyl-N-acetylcysteine; and carbon dioxide, 1,2-ethanediamine, glycine, and S-(succin-2-yl)-N-acetylhomocysteine. The conjugates, Ps-A'-S-E'-B'-Pro, according to this invention, may contain spacers whose components include derivatives of, *inter alia*: carbon dioxide and S-carboxymethylcysteamine; carbon dioxide and S-(α-carboxyethyl)cysteamine; carbon dioxide and S-carboxymethylhomocysteamine; carbon dioxide, S-(succin-2-yl)cysteamine, and glycine; and carbon dioxide and S-carboxymethylcysteine.

35 In the process of the present invention, the polysaccharide is covalently-modified by (a) solubilizing it in a non-hydroxylic organic solvent, then (b) activating it with a bifunctional reagent, (c) reacting this activated polysaccharide with a bis-nucleophile, and finally, if necessary, further (d) functionalizing this modified polysaccharide by either reaction, (i) with a reagent generating electrophilic (e.g., thiophilic) sites or, (ii) with a reagent generating thiol groups. The protein is conversely either reacted (i) with a reagent generating thiol groups or (ii) with a reagent generating thiophilic sites, then the covalently-modified polysaccharide and the functionalized protein are reacted together to form the stable covalently-bonded conjugate and the final mixture is purified to remove unreacted polysaccharides and proteins.

The process of this invention also includes selection of a nucleophile or bis-nucleophile which will react with the activated polysaccharide to form a covalently-modified polysaccharide with pendant electrophilic sites or pendant thiol groups, thereby obviating the need to further functionalize the bis-nucleophile-modified polysaccharide prior to reacting the covalently-modified polysaccharide with the covalently-modified protein. Also, the functionalization of the protein to either moiety form may be accomplished in more than one step according to the selection of reactants in these steps.

In the first step toward covalently-modifying the polysaccharide, the solid polysaccharide must be solubilized.

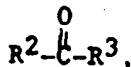
Since the nucleophilic alcoholic hydroxyl groups of a polysaccharide cannot compete chemically for electrophilic reagents with the hydroxyls of water in an aqueous solution, the polysaccharide should be dissolved in non-aqueous (non-hydroxylic) solvents. Suitable solvents include dimethylformamide, dimethylsulfoxide, dimethylacetamide, formamide, N,N'-dimethylimidazolidinone, and other similar polar, aprotic solvents, preferably dimethylformamide.

In addition to the use of these solvents, converting the polysaccharides (e.g., the capsular polysaccharides of *H. influenzae* type b, which are a ribose-ribitol phosphate polymers), which have acid hydrogens, such as phosphoric acid mono- and diesters, into an appropriate salt form, causes the polysaccharides to become readily soluble in the above solvents. The acidic hydrogens in these macro-molecules may be replaced by large hydrophobic cations, such as tri- or tetra-(C₁- to C₆)alkyl-ammonium, 1-azabicyclo[2.2.2]octane, 1,8-diazabicyclo[5.4.0]undec-7-ene or similar cations, particularly tri- or tetra-(C₁- to C₆)alkylammonium, and the resultant tri- or tetraalkylammonium or similar salts of phosphorylated polysaccharides readily dissolve in the above solvents at about 17°-50°C, while being stirred for from one minute to one hour.

Partially-hydrolyzed *H. influenzae* serotype B polysaccharide has been converted into the tetrabutyl-ammonium salt, then dissolved in dimethylsulfoxide (Egan et al., *J. Amer Chem. Soc.*, 104, 2898 (1982)), but this product is no longer antigenic, and therefore useless for preparing vaccines. By contrast, Applicants accomplish the solubilization of an intact, unhydrolyzed polysaccharide by passing the polysaccharide through a strong acid cation exchange resin, in the tetraalkylammonium form, or by careful neutralization of the polysaccharide with tetraalkyl-ammonium hydroxide, preferably by the former procedure, and thereby preserve the viability of the polysaccharide for immunogenic vaccine use.

Subsequent steps are then directed to overcoming the other significant physico-chemical limitation to making covalent bonds to polysaccharides, that being the lack of functional groups on the polysaccharides, other than hydroxyl groups, which are reactive enough with reagents commonly or practically used for functionalization of units with which bonding is desired. Activation of the polysaccharide to form an activated polysaccharide, reaction with bis-nucleophiles to form a nucleophile-functionalized polysaccharide, and functionalization with reagents generating either electrophilic sites or thiol groups, are all directed to covalently-modifying the polysaccharide and developing functional groups on the polysaccharide in preparation for conjugation.

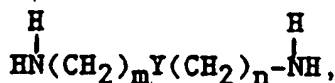
In the next step, the solubilized polysaccharide is activated by reaction with a bifunctional reagent at about 0°-50°C, while stirring for ten minutes to one hour, with the crucial weight ratio of activating agent to polysaccharide in the range of 1:5 to 1:12. In the past, this activation has been accomplished by reaction of the polysaccharide with cyanogen bromide. However, derivatives activated with cyanogen bromide, which has a "proclivity" for vicinal diols, have shown transient stability during dialysis against a phosphate buffer. Therefore, while activation with cyanogen bromide is still possible according to the present invention, this reagent is poorly utilized in activation of polysaccharides and is not preferred. Instead, preferred bifunctional reagents for activating the polysaccharide include carbonic acid derivatives,



wherein R² and R³ may be independently, azolyl, such as imidazolyl; halides; or phenyl esters, such as p-nitrophenyl, or polyhalophenyl.

Carbonyldiimidazole, a particularly preferred reagent, will react with the hydroxyl groups to form imidazolylurethanes of the polysaccharide, and arylchloroformates, including, for example, nitrophenylchloroformate, will produce mixed carbonates of the polysaccharide. In each case, the resulting activated polysaccharide is very susceptible to nucleophilic reagents, such as amines, and is thereby transformed into the respective urethanes.

In the next stage, the activated polysaccharide is reacted with a nucleophilic reagent, such as an amine, particularly diamines, for example,



5 wherein m is 0 to 4, n is 0 to 3, and Y is CH₂, O, S, NR', CHCO₂H, where R' is H or a C₁- or C₂-alkyl, such that if Y is CH₂, then both m and n cannot equal zero, and if Y is O or S, then m is greater than 1, and n is greater than 1, in a gross excess of amine (i.e., for example, a 50-to 100-fold molar excess of amine vs. activating agent used). The reaction is kept in an ice bath for from 15 minutes to one hour then kept for 15 minutes to one hour at about 17° to 40°C.

10 An activated polysaccharide, when reacted with a diamine, e.g., 1,4-butanediamine, would result in a urethane-form polysaccharide with pendant amines, which may then be further functionalized by acylating. Mixed carbonates will also readily react with diamines to result in pendant amine groups.

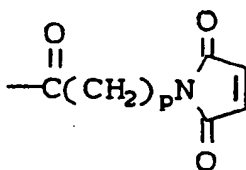
Alternatively, the activated polysaccharide may be reacted with a nucleophile, such as a mono-haloacetamide of a diaminoalkane, for example, 4-bromoacetamidobutylamine (see, W. B. Lawson *et al.*,
15 Hoppe Seyler's *Z. Physiol Chem.*, 349, 251 (1968)), to generate a covalently-modified polysaccharide with pendant electrophilic sites. Or, the activated polysaccharide may be reacted with an aminothiols, such as cysteamine (aminoethanethiol) or cysteine, examples of derivatives of which are well-known in the art of peptide synthesis, to produce a polysaccharide with pendant thiol groups. In both cases, no additional functionalization is necessary prior to coupling the covalently-modified polysaccharide to the modified bacterial "carrier" protein.

20 The last step in preparing the polysaccharide, the further functionalization, if necessary, of the polysaccharide, may take the form of either reacting the nucleophile-functionalized polysaccharide with a reagent to generate electrophilic (i.e., thiophilic) sites, or with a reagent to generate thiol groups.

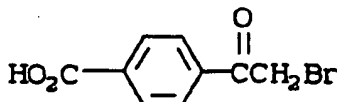
Reagents suitable for use in generating electrophilic sites, include for example, those for acylating to α-haloacetyl or α-halopropionyl, derivative such as



30 (wherein R is H or CH₃; X is Cl, Br or I; and X' is nitrophenoxy, dinitrophenoxy, pentachlorophenoxy, pentafluorophenoxy, halide, O-(N-hydroxysuccinimidyl) or azido), particularly chloroacetic acid or α-bromopropionic acid, with the reaction being run at a pH of 8 to 11 (maintained in this range by the addition of base, if necessary) and at a temperature of about 0° to 35°C, for ten minutes to one hour. An amino-derivatized polysaccharide may be acylated with activated maleimido amino acids (see, O. Keller *et al.*, *Helv. Chim. Acta.*, 58, 531 (1975))
35 to produce maleimido groups,

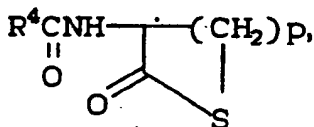


45 wherein p is 1 to 3; with a 2-haloacetylating agent, such as p-nitrophenylbromoacetate; or with an α-haloketone carboxylic acid derivative, e.g.,

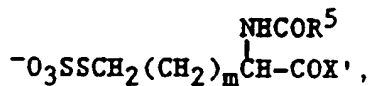


50 (Ber., 67, 1204, (1934)) in order to produce appropriately functionalized polysaccharides susceptible to thiosubstitution.
55

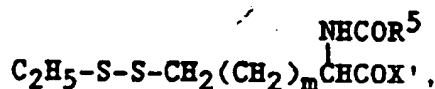
Reagents suitable for use in generating thiol groups include, for example, acylating reagents, such as thiolactones, e.g.,



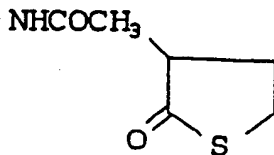
wherein R⁴ is C₁- to C₄-alkyl or mono- or bicyclic aryl, such as C₆H₅ or C₁₀H₁₃, and p is 1 to 3;



wherein m is 0 to 4, R⁵ is C₁- to C₄-alkyl or C₆H₅, and X' is as defined above, followed by treatment with HSCH₂CH₂OH; or

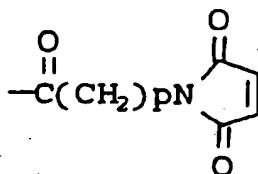


wherein m, R⁵ and X' are as defined immediately above, then treatment with dithiothreitol. Such reactions are carried out in a nitrogen atmosphere, at about 0° to 35°C and at a pH of 8 to 11 (with base added, as necessary, to keep the pH within this range), for one to twenty-four hours. For example, an amino-derivatized polysaccharide may be reacted with



to produce an appropriately-functionalized polysaccharide.

By these steps then, covalently-modified polysaccharides of the forms, Ps-A-E* or Ps-A'-SH-, wherein E* is -CCHX or

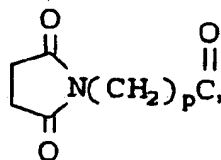


and A, A', R, X and p are as defined above, are produced.

Separate functionalization of the protein to be coupled to the polysaccharide, involves reaction of the protein with one or more reagents to generate a thiol group, or reaction of the protein with one or more reagents to generate an electrophilic (i.e., thiophilic) center.

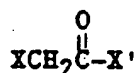
In preparation for conjugation with an electrophilic-functionalized polysaccharide, the protein is reacted in one or two steps with one or more reagents to generate thiol groups, such as those acylating reagents used for generating thiol groups on polysaccharides, as discussed on pages 15-17 above. Thiolated proteins may also be prepared by aminating carboxy-activated proteins, such as those shown in Atassi *et al.*, *Biochem et Biophys Acta*, 670, 300, (1981), with amino thiols, to create the thiolated protein. A preferred embodiment of this process step involves the direct acylation of the pendant amino groups (i.e., lysyl groups) of the protein with N-acetylhomocysteinethiolactone at about 0° to 35°C and pH 8-11, for from five minutes to two hours, using equiweights of reactants.

When $E'B'$ is

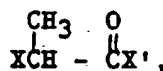


10 the conditions and method of preparing the functionalized protein are as discussed above for preparing the counterpart polysaccharide by reaction with activated maleimido acids.

In preparing for conjugation with a covalently-modified bacterial polysaccharide with pendant thiol groups, the protein is acylated with a reagent generating an electrophilic center, such acylating agents including, for example,

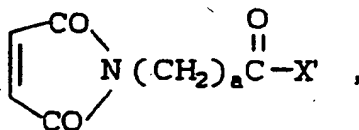


20 and

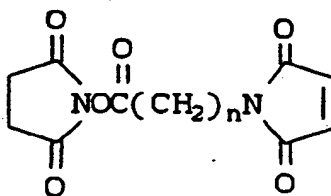


25

wherein X and X' are as defined above; and



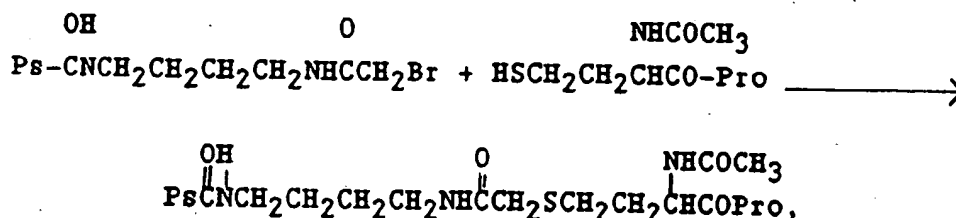
wherein X' is as defined above. Suitable proteins with electrophilic centers also include, for example, those prepared by acylation of the pendant lysyl amino groups with a reagent, such as activated maleimido acids, for example,



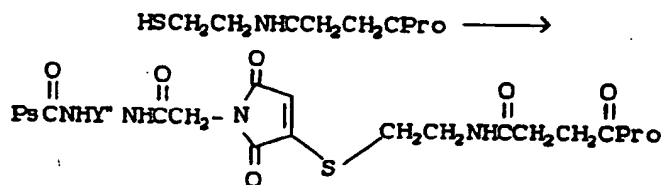
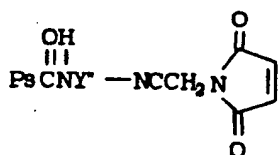
45

or by reacting the carboxy-activated protein with monohaloacetyl derivatives of diamines. In both preparation reactions, the temperature is from 0° to 35°C for from five minutes to one hour and the pH is from 8 to 11.

Formation of the conjugate is then merely a matter of reacting any of the covalently-modified polysaccharides having pendant electrophilic centers with of the bacterial protein MIEP having pendant thiol groups at a pH of 7 to 9, in approximate equiweight ratios, in a nitrogen atmosphere, for from six to twenty-four hours at from about 17° to 40°C, to give a covalent conjugate. Examples of such reactions include:

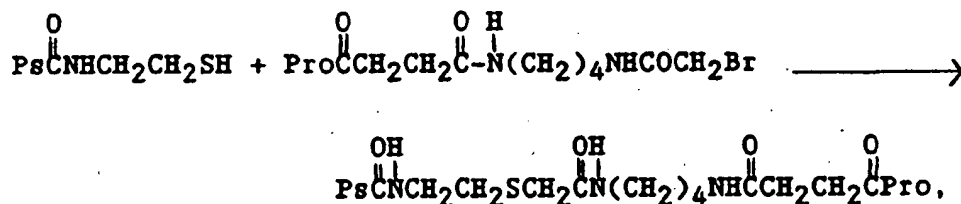


wherein an activated polysaccharide which has been reacted with 4-bromoacetamidobutylamine is reacted with a protein which has been reacted with N-acetyl-homocysteinethiolactone, to form a conjugate, and:



(where Y* is a C₂-C₈alkyl radical), wherein an amino-derivatized polysaccharide which has been reacted with activated maleimido acids is reacted with a carboxy-activated protein which has been aminated with an aminothiol, to form a conjugate.

Similarly, any of the covalently-modified polysaccharides with pendant thiol groups may be reacted with the bacterial protein MIEP having pendant electrophilic centers to give a covalent conjugate. An example of such a reaction is:



wherein an activated polysaccharide which has been reacted with an aminothiol is reacted with a carboxy-activated protein which has been reacted with monohaloacetyl derivatives of a diamine, to form a conjugate.

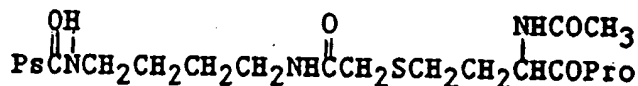
Should the electrophilic activity of an excess of haloacetyl groups need to be eliminated, reaction of the conjugate with a low molecular weight thiol, such as n-acetylcysteine, will accomplish this purpose. Use of this reagent, n-acetylcysteine, also allows confirmation accounting of the haloacetyl moieties used (see Section D), because the S-carboxymethylcysteine which is formed may be uniquely detected by the method of Spackman, Moore and Stein.

These conjugates are then centrifuged at about 100,000 x g using a fixed angle rotor for about two hours at about 1° to 20°C, or are submitted to any of a variety of other purification procedures, including gel permeation, ion exclusion chromatography, gradient centrifugation, or other differential adsorption chromatography, to remove non-covalently-bonded polysaccharides and proteins, using the covalency assay for the bigenic spacer (see below) as a method of following the desired biological activity.

The further separation of reagents may be accomplished by size-exclusion chromatography in a column, or in the case of very large, non-soluble proteins, separation may be accomplished by ultracentrifugation.

Analysis of the conjugate to confirm the covalency, and hence the stability of the conjugate, is accomplished

by hydrolyzing (preferably with 6N HCl at 110°C for 20 hours) the conjugate, then quantitatively analyzing for the amino acid of the hydrolytically-stable spacer containing the thioether bond and constituent amino acids of the protein. The contribution of the amino acids of the protein may be removed, if necessary, by comparison with the appropriate amino acid standard for the protein involved, with the remaining amino acid value reflecting the covalency of the conjugate, or the amino acid of the spacer may be designed to appear outside the amino acid standard of the protein in the analysis. The covalency assay is also useful to monitor purification procedures to mark the enhancement of concentration of the biologically active components. In the above examples, hydrolysis of



results in the release of S-carboxymethylhomocysteine,

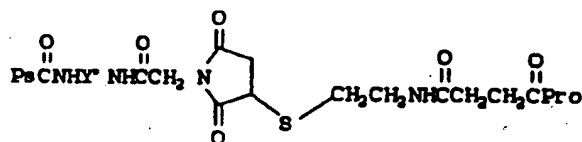


hydrolysis of

In mice this requirement exists for secondary, as well as primary, antibody responses and is carrier-specific, i.e. a secondary antibody response occurs only if the T helper cells have previously been sensitized with the carrier protein used for the secondary immunization. Therefore, the ability of a mouse to make a secondary antibody response to a PRP-protein conjugate is dependent on the presence of primed T lymphocytes with specificity for the carrier protein.

Demonstration of the ability of MIEP to provide carrier priming for anti-PRP antibody responses was done in mice adoptively primed with PRP covalently linked to a heterologous carrier, diphtheria toxoid (DT). Adoptive transfer was used in order to determine whether the administration of lymphocytes primed with MIEP alone was sufficient to generate effective helper-T cell activity for anti-PRP antibody formation in response to PRP-OMPC. Comparable secondary anti-PRP antibody responses were elicited by PRP-OMPC when lymphocytes primed with MIEP or OMPC were transferred, indicating that the T cell recognition of OMPC resides in the MIEP moiety.

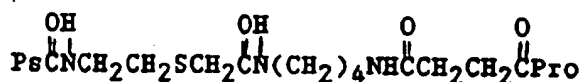
PRP-MIEP conjugates were tested for immunogenicity in mice as well as infant Rhesus monkeys. The immune response in both of these animal models share, with infant humans, a deficiency in their ability to generate antibody responses against T-independent antigens such as bacterial polysaccharides. These animals are commonly used as models for assessment of the immune response of infant humans to various antigens.



results in the release of the aminodicarboxylic acid,



and hydrolysis of



results in the release of S-carboxymethylcysteamine, $\text{H}_2\text{NCH}_2\text{CH}_2\text{SCH}_2\text{CO}_2\text{H}$ by cleavage of the Ps-A-E-S-B-Pro molecule at peptide linkages and other hydrolytically-unstable bonds. Chromatographic methods, such as

those of Spackman, Moore, and Stein, may then be conveniently applied and the ratio of amino acid constituents determined.

Optimal production of IgG antibody requires collaboration of B and T lymphocytes with specificity for the antigen of interest. T lymphocytes are incapable of recognizing polysaccharides but can provide help for anti-polysaccharide IgG antibody responses if the polysaccharide is covalently linked to a protein which the T cell is capable of recognizing.

Likewise, MIEP-peptide conjugates, for example where the peptide is an HIV principal neutralizing determinant (PND) peptide, may be prepared. One method of making such conjugates includes the formation of a bigeneric spacer between activated MIEP and activated HIV PND peptides. The linker may include a polysaccharide moiety.

The novel conjugate of this invention comprises MIEP, the major immuno enhancing protein of the outer membrane protein complex (OMPC) of *Neisseria meningitidis* b, covalently linked to HIV PND peptides.

The conjugates are prepared by the process of covalently coupling activated peptide to an activated protein. The peptide and protein components are separately activated to display either pendant electrophilic or nucleophilic groups so that covalent bonds will form between the peptide and the protein upon contact.

The covalent conjugate immunogens that result from the series of reactions described above may conveniently be thought of as a conjugate in which multiple peptide functionalities are built upon a foundation of MIEP.

When the peptide components of the conjugate are capable of eliciting HIV neutralizing immune responses, the conjugates of this invention may be administered to mammals in immunologically effective amounts, with or without additional immunomodulatory, antiviral, or antibacterial compounds, and are useful for inducing mammalian immune responses against the peptidyl portion of the conjugates, for inducing HIV-neutralizing antibodies in mammals, or for making vaccines for administration to humans to prevent contraction of HIV infection or disease including AIDS, or for administration to humans afflicted with HIV infection or disease including AIDS.

In a preferred embodiment, the conjugate of the invention has the general structure:



or pharmaceutically acceptable salts thereof, wherein:

PEP is an HIV PND peptide, or a peptide capable of raising mammalian immune responses which recognize HIV PNDs;

MIEP is an immunogenic protein of the outer membrane protein complex (OMPC) of *Neisseria meningitidis* b either recombinantly produced or purified from OMPC;

-A- is a covalent linkage, preferably a bigeneric spacer;

j is the percentage by mass of peptide in the coconjugate, and is preferably between 1% and 50% of the total protein mass in the conjugate.

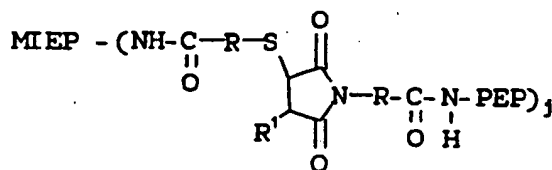
The conjugate of the invention may be prepared by any of the common methods known in the art for preparation of peptide-protein conjugates, such as, for example, the bigeneric chemistry disclosed in U.S. patent 4,695,624 and Marburg et al. J.A.C.S. 108, 5282 (1986), and in Applications USSN 362,179; 55,558; 55,974; 55,966 and 55,339. In a preferred embodiment, a process that utilizes the available nucleophilic functionalities, found in proteins, such as the amino group of lysine, the imidazole group of histidine, or the hydroxyl groups of serine, threonine, or tyrosine is used. In practical terms, the number of available protein nucleophilic sites may be determined by an appropriate assay which may comprise thiolation with N-acetyl homocysteine thiolactone, followed by Ellman Assay [Ellman, G.L., *Arch Biochem Biophys.*, 82, 70 (1959)] for determination of total free sulfhydryl groups and/or by alkylation with a bromoacetyl amino acid, assayable by amino acid analysis.

The preferred process can be carried out in several ways in which the sequence, method of activation, and reaction of protein and peptide groups can be varied. The process may comprise the steps of:

Process 1:

1a. reacting the protein nucleophilic groups with a reagent, for example with N-acetyl homocysteine thiolactone, which generates thiol groups on the protein; and

1b. reacting the product of step 1a. with peptides previously derivatized so as to append an electrophilic group preferably comprising maleimide, on the peptide. A preferred embodiment of this invention, which may be prepared according to this process, has the structure:



or pharmaceutically acceptable salts thereof, wherein:

PEP, MIEP, and j , are as defined supra;

-R- is:

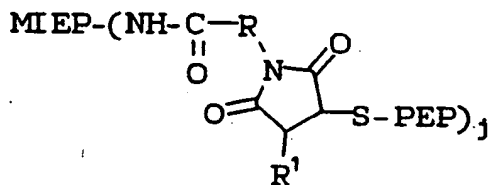
- a) -lower alkyl-,
- b) -substituted lower alkyl-,
- c) -cycloalkyl-,
- d) -substituted cycloalkyl-,
- e) -phenyl-;

-R¹ is:

- a) -hydrogen,
- b) -lower alkyl, or
- c) -SO₃H; and

-S- is sulfur

Likewise, a preferred embodiment of the invention having the structure:



wherein all variables are as defined above, may be prepared by process 2, which comprises the steps of:

2a. reacting the protein nucleophilic groups with a bifunctional electrophilic reagent, such as maleimidoalkanoic acid hydroxysuccinimide ester, so as to generate an electrophilic protein; and

2b. reacting the product of step 2a. with a peptide containing a nucleophile, such as a thiol group.

A highly preferred embodiment of process 1, is described in detail below and in Scheme A. According to the scheme, the immunogenic protein is the class II protein of the outer membrane protein complex (OMPC) of *Neisseria meningitidis* b, either purified from the bacterial membrane or produced by recombinant means. The process comprises the steps of:

a.i. reacting MIEP (I), having nucleophilic groups, including free amino groups due to the presence of lysines or protein amino-termini, with a thiolating agent, preferably N-acetyl homocysteine thiolactone, to generate MIEP (II) having "m" moles of sulfhydryl groups available for reaction with a thiophile; a.ii. quantitating the number of available sulfhydryls appended to MIEP in step 1a.i. to determine the value of "m", preferably by Ellman assay (Ellman, G.L., *Arch. Biochem. Biochem. Biophys.*, 82, 70 (1959)); and b. contacting the product of step a. with an excess, ($\geq m$), of an HIV PND which has been previously derivatized so as to append an electrophilic group, preferably with a maleimido-alkanoic acid, and most preferably with maleimido-propionic acid (this derivatization is achieved by N-protecting all amino groups on the peptide that should not be derivatized, and reacting the free peptide amino groups with a bifunctional reagent, preferably maleimidoalkanoxyloxysuccinimide, and most preferably maleimidopropionyloxysuccinimide), to generate the conjugate of this invention (III).

The conjugate product may be purified by, for example, dialysis in a buffer having an ionic strength between 0.001M and 1M and a pH between 4 and 11, and most preferably in an aqueous medium having an ionic strength of between 0.01 and 0.1M and a pH of between 6 and 10.